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Neurofibromatosis is a genetic disease in which the major pathology is due to abnormal proliferation of Schwann cells. One of the two tumor suppressor genes mutated in this disease is the neurofibromatosis 1 gene (NF1). The product of this gene, neurofibromin, inhibits *ras* function. Based on several unique features of control of growth in Schwann cells, we believe that specific factors associated with CREB, and the CREB-binding protein, CBP, serve to specifically modulate cAMP and *ras* -dependent growth events in glia, and underly the NF1 mutation-induced Schwann cell proliferation. Our identification of CBP as an integral component of the activation complex for cAMP and Growth factors, and evidence that competition for limiting amounts of CBP can result in AP-1 inhibiting events, has led us to provide evidence for a molecular mechanism which integrates diverse signaling pathways, dictating differentiation and proliferation events. We hypothesize that this regulatory system can be exploited to discover novel approaches to the treatment of neurofibromatosis. We have proposed to generate various genetic models exhibiting altered cAMP and *ras* -dependent signaling pathways and utilize these model systems to identify and characterize factors which are responsible for the etiology of neurofibromatosis type 1.

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TABLE OF CONTENTS

INTRODUCTION

SUBJECT

Neurofibromatosis is an inherited genetic disorder that results from mutations in either of two apparent tumor suppressor genes. The most common version of the disease, neurofibromatosis type 1, (Von Recklinghausen neurofibromatosis) is a human neoplastic syndrome with a prevalence of 1:3500, due to alterations in the neurofibromatosis 1 gene (*NF1*). The predominant abnormalities associated with neurofibromatosis type 1 are the clonal expansions of neural crest derived tissues, particularly Schwann cells and melanocytes. Neurofibromas are composed primarily of Schwann cells; malignant neurofibrosarcoma, astrocytoma, pheochromocytoma, Wilms tumor, leukemia and embryonal rhabdomyosarcoma occur at rates significantly higher than in the general population, but in < 5% of NF1 patients. The disease is most often treated with surgical removal of the usually benign cutaneous tumors, but more internally occurring neurofibromas can intrude on nearby tissues and also have a low but significant risk of acquiring additional genetic alterations during proliferation that result in malignancies. Approximately one third of those affected experience symptoms more serious than can be addressed by routine surgery. There is at present no cure for neurofibromatosis, and the premise of our application is that, by better understanding of the molecular mechanisms that underlie the normal molecular mechanisms that regulate Schwann cell division, and the basis of the aberrant proliferation of these cells, that we can devise strategies that will ultimately lead to practical treatments.

The product of the neurofibromatosis type I gene, neurofibromin, appears to inhibit *ras* function, a cytoplasmic proto-oncogene gene product normally associated with control of cellular growth and proliferation, and abnormally up-regulated in many models of unrestricted cellular growth, but abnormal proliferation in neurofibromatosis type I is limited to a few cell types, including Schwann cells. Because Schwann cells have unusual regulation of growth, including stimulation by an intracellular "second messenger," cAMP, that usually inhibits growth in many other cell types, there is an opportunity to devise a cell-specific strategy. cAMP works largely by activating gene expression, working through a specific transcription factor called the cAMP response element binding protein (CREB). Based on several unique features of control of growth in Schwann cells, we believe that specific factors associated with CREB, and the CREB-binding protein, CBP, serve to specifically modulate cAMP and *ras* -dependent growth events in glia, and underly the NF1 mutation induced Schwann cell proliferation. Our identification of CBP as an integral component of the activation complex for nuclear receptors, as well as for cAMP and growth factors, and evidence that competition for limiting amounts of CBP can result in AP-1 inhibiting events, has led us to suggest and provide evidence for a molecular mechanism by which one can integrate diverse signaling pathways, specifying differentiation and proliferation events.

PURPOSE

We suggest that this unique regulatory system for the control of proliferation in Schwann cells can be exploited to discover novel approaches to the treatment of neurofibromatosis type I. We have proposed and have initiated studies directed to further understand normal Schwann cell development and the role of *NF1* mutations in the abnormal proliferation of Schwann cells. While the ability of *NF1* to inhibit *ras* function appears to be well-established, the proposed studies may define the molecular basis of the abnormal

proliferation at the transcriptional level, potentially leading to novel therapeutic approaches. We have therefore hypothesized that in myelinating glia of the central and peripheral nervous system, there will be a specific modulation of CBP function that underlies the cAMP-dependent and *ras*- dependent growth events in glia, and that misdirection of this modulation as a consequence of improper regulation of the *ras* -signaling pathway by mutant or absent neurofibromin underlies the NF1 mutation-induced proliferation. Our proposed experiments are designed to identify the factors expressed as a consequence of these misdirected programs of gene transcription caused by redirected nuclear allocation of CBP in the mediation of nuclear transcription. It is likely that the groups of gene products identified as a result of aberrant integration of second messenger signaling pathways through CBP-directed gene expression will contain targets to which novel therapeutic approaches to neurofibromatosis might be applied.

SCOPE

We have proposed to identify and functionally evaluate target genes responsible for the abnormal proliferation of Schwann cells which characterize the phenotype of neurofibromatosis type I. Our hypothesis that altered integration of intracellular signaling pathways at the nuclear level, due to increased competition for the signaling integrator CBP by nuclear factors such as AP-1, leads to abnormal proliferative events, suggests that the characterization of gene products, the expression of which are either up-regulated or down-regulated by these misdirected signaling events, would be instrumental in the identification of factors responsible for the abnormal proliferation of Schwann cells. Therefore, we have undertaken to identify and successfully develop a reliable procedure, termed "representational difference analysis" (RDA) for the identification of such factors by exploiting the differences in the expression of target genes between two populations of otherwise identical cells, in one of which intracellular signaling is normal, as is the case for wild-type cells, and in the other cellular signaling is misregulated, as has been demonstrated for cells carrying the *NF1* mutation. In the process of establishing the "proof of principle" of this methodology we have utilized for these purposes two distinct pairs of cell populations, both of which are of the Schwann cell lineage.

In a parallel approach, we have undertaken the mapping of a segment of the mouse *NF1* locus and the construction of a targeting vector as part of an approach to develop representative models of *NF1* mutations in mice using the Cre Lox-P system to introduce an *NF1* mutation consisting of the deletion of exon 31, which will be conditionally deleted in glial cells, but not elsewhere, thereby avoiding the early embryonic lethal phenotype associated with existing germline mutations of the NF-1 gene, such that Schwann cells from these tissues can be utilized in concert with wild-type Schwann cell tissues in RDA analyses designed to identify gene products either over- or under-expressed in these Schwann cell populations. This mutation will result in a termination codon nine amino acids downstream of the splice junction between exons 30 and 32, thus creating an effective disruption in the GAP-related homology domain implicated in neurofibromin modulation of the ras signaling pathway. Generation of this mutated version of the *NF1* gene provides us with the most optimal approach to ultimately obtain Schwann cells exhibiting the neurofibromatosis type I etiology, whereby the normal neurofibromin regulation of the ras/AP1 pathway is disrupted, thus insuring a promising result upon application of the RDA technology to obtaining downstream target genes, the mis-regulated expression of which is responsible for the neurofibromatosis type I phenotype.

Furthermore, a strategy in process to create transgenic mice harboring a dominant negative CREB mutant, incapable of functional induction by cAMP, and expressed specifically in glial cells, will permit the analysis of Schwann cells that are compromised in their abilities to respond to cAMP as compared to wild-type counterparts. RDA analysis of this CREB mutation

bred into the genetic background of the *NF1* mutation described above will permit isolation of Schwann cells for which the balance of signal integration is even further shifted towards the throughput of ras/AP1, and their analysis will further elucidate the nature of the classes of target genes induced by the proper regulation of the ras/AP-1 signaling pathway by NF 1. Additionally, functional analysis of nuclear regulation of CBP by cAMP/ras signaling pathways is under investigation utilizing a study of CBP interaction with nuclear cofactors through interactions with the LXXLL domain we have recently identified as shared by these factors (Torchia et al., 1997).

In order that any putative target genes might be examined for functional effects on Schwann cell proliferation, we have proposed to adapt a microinjection strategy, currently in use in our laboratory with various cell line models, for use in primary cultures of Schwann cells. Consequently, we have undertaken to establish the tissue culture conditions required for the cultivation of primary cultures of Schwann cells, and have established parameters required for the microinjection of these cells with antibodies and plasmid expression vector systems in anticipation of future evaluation of various target genes.

BACKGROUND OF PREVIOUS WORK

The characterization of factors required for the integration of diverse intracellular signaling pathways resulting in the proliferative response has been studied extensively. Our studies over the past several years have set the experimental premise for the proposed investigation of normal and disordered regulation of growth of myelinating glia. References pertinent to the background of our previous work include: Horlein et al., 1995; Schonemann et al., 1995; Bermingham et al., 1996; Erkman et al., 1996; Kamei et al., 1996; McEvilly et al., 1996; Torchia et al. 1997. In addition to Schwann cells, several other cell types, including pituitary somatotropes, thyroid exocrine cells, and parotid gland cells, exhibit cAMP-dependent increases in proliferation (Ambesi-Impiombato et al., 1980; Tsang et al., 1980; Roger et al., 1983; Levine et al., 1988; Weinstein et al., 1991; Hen et al., 1989). In the case of the pituitary somatotropes, increased proliferation is blocked by a dominant-negative mutation in the cAMP response element binding protein (CREB, Ser 133→Ala) (Struthers et al., 1991), indicating that both activation by cAMP-dependent protein kinase, as well as activation of ras pathways, is required for proliferation effects. Recently, we have determined that a factor required for CREB, the CREB binding protein (CBP), serves as an integrator that interacts with, and mediates the function of, a series of transcription factors, including classes of nuclear receptor transcription factors, and we wish to determine the roles of these factors in Schwann cell growth and development.

The 265 kDa CREB binding protein and the related protein, p300, thus appear to be required for effective ligand-dependent gene activation events by nuclear receptors (Kamei et al., 1996; Yao et al., 1996; Chakravarti et al., 1996; Horvai et al. 1996; Bhattacharya et al., 1996). A crucial role of CBP in ligand-dependent activation events is supported by multiple independent types of evidence, including the observations that ligand-dependent association between CBP and nuclear receptors occurs both in intact cells and on DNA-bound receptor complexes and that anti-CBP antibodies selectively inhibit the transcriptional activities of nuclear receptors in intact cells. Thus, all CBP function is linked to transactivation by CREB, Jun/Fos, and nuclear receptors, and we have obtained recent evidence that shows requirement for the JAK/STAT pathway-dependent transcriptional activation (Horvai et al., 1996; Bhattaacharya et al., 1996).

CBP Serves as a Nuclear Co-Integrator

We established that direct interactions occurred between nuclear receptors and CBP using a series of overlapping fragments of the 265 kDa CBP protein that were bacterially expressed as glutathione-S-transferase (GST) fusion proteins, revealing a strong ligand-dependent interaction confined to a single N-terminal CBP region. The ability of this interaction to occur in intact cells in a ligand-dependent manner was confirmed using the yeast two-hybrid system. Mapping revealed that the N-terminal 100 amino acids of CBP retained full interaction with retinoic acid receptor; attempts to further subdivide this region resulted in a complete loss of capacity to interact. Consistent with the ability of several nuclear receptors to inhibit the activated AP-1 transcriptional response, we found that estrogen, thyroid hormone, and retinoid X receptors, also exhibited strong ligand-dependent interactions, with the same N-terminal domain of CBP, dependent upon a functional AF2 domain, and these interactions were demonstrated to occur on DNA. Several assays established that ligand-dependent interactions between retinoic acid or glucocorticoid receptors and CBP occurred *in vivo.*

Based on evidence in co-transfection assays and *in vitro* transcription assays suggesting that CBP could increase nuclear receptor function, the role of CBP in ligand-dependent transcriptional activation was directly documented by evaluating the ability of microinjected anti-CBP IgG to inhibit ligand-dependent transcription from a retinoic acid response element-containing promoters in intact cells. Co-injection of increasing concentrations of purified anti-CBP IgG, but not control IgG, resulted in a progressive and specific decrease in ligand-dependent gene activation, producing >80% inhibition of ligand-induced activation, and reduced reporter expression in the remaining 20% of cells that still scored positive (Kamei et al., 1996).

Therefore, we investigated the possibility that the major biochemically-identified putative nuclear receptor co-activators, p140 or p160, could themselves interact with CBP, identifying specific interaction between the C-terminal region of CBP and p160 , but not with p140. These regions are distinct from the internal regions of CBP that interact with CREB, Fos, TFIIB, and E1A (Chrivia et al., 1993; Kwok et al., 1994; Arany et al., 1995; Bannister and Kouzarides, 1995). A region of 105 amino acids was sufficient for ligand-dependent interactions with p160 (Kamei et al., 1996).

cDNAs encoding the putative p160 were obtained by expression cloning based on the criteria that phage plaques exhibited interaction with both the CBP C-terminus and liganded nuclear receptors, revealing a family of three related genes, encoding variant forms of SRC-1 (Oñate et al., 1995), and two novel forms. The N-terminal sequence of all three exhibited a homology to the bHLH, A/B regions of the PAS domain characteristic of the PAS/HLH gene family. Antisera raised against an internal 50 kDa region common to all the recombinant SRC-1 variant proteins specifically interacted with the 160 kDa protein doublet in whole cell extracts, and by immunodepletion, approximately 75% of the biochemically-defined p160 could be removed from whole cell extracts by this antiserum. These data indicate that the cloned factors represent the biochemically-identified p160 (Kamei et al., 1996).

While p300 was initially described based on its interaction with E1A (Eckner et al., 1994), recent data confirmed its ability to interact with both CREB and Jun/Fos; conversely, CBP interacts with E1A (Arany et al., 1995). Although not initially recognized as regions of high homology, the N- and C-terminal regions of CBP and p300 revealed multiple clusters of complete identity in both the nuclear receptor interaction domain (amino acids 1-101, CBP, and amino acids 1-117, p300) and in the p160 interaction domain (amino acids 2058-2163, CBP; amino acids 2042-2157, p300). Consistent with these sequence homologies, the N-terminus of p300 interacted with the retinoic acid receptor in a ligand-dependent manner, while its C-

terminus interacted with p160. The strength of these interactions was comparable to those of the corresponding regions of CBP (Kamei et al., 1996).

To test the role of p160 in nuclear receptor activity, specific antisera were raised against bacterially-expressed p160 protein, and the IgG were purified. By microinjection assay, we find that one factor, that we refer to as N-CoA 1, is required for nuclear receptor activation function.

Role of CBP in Nuclear Receptor-Mediated Inhibition of AP-1 Activity

We explored a potential role for CBP in mediation of ligand-dependent inhibition of AP-1 function, to test whether competition for limiting quantities of CBP accounts for the inhibitory effect of nuclear receptors. Indeed, the inhibitory effect of liganded retinoic acid and glucocorticoid receptors were largely or completely abolished by co-transfection of vectors expressing CBP or p300. Mutations in the retinoic acid receptor AF2 domain that inhibit binding of CBP and other co-activator proteins abolished AP-1 repression by nuclear receptors, consistent with previous data involving C-terminal receptor deletions.

These observations are consistent with the hypothesis that the interaction between retinoic acid receptors and CBP is responsible for some forms of repression of AP-1 activity. If this were the case, one would predict that CREB, which appears to have a very high affinity for CBP, might be able to actually inhibit nuclear receptor activation. Indeed, we found that activated CREB reduced retinoic acid-dependent stimulation from >40-fold to ~5 fold, without affecting the basal activity of the RARE-driven reporter (Kamei et al., 1996).

The identification of CBP as an integral component of the activation complex for nuclear receptors obviously suggests a molecular mechanism by which nuclear receptors might inhibit AP-1 action, based on a requirement for the relatively low intracellular levels of the CBP/p300 family proteins by both classes of transcription factors. Because increasing the intracellular levels of this family of co-activators serves to abolish retinoic acid or glucocorticoid receptor repression of AP-1, and because AP-1 can effectively bind to RAR-associated CBP, it is likely that the limiting amount of the CBP family factors is partitioned by the alternative activation of nuclear receptors by ligand, or of AP-1 by phosphorylation. Although a competition model provides a mechanism for the observed antagonism between nuclear receptors and AP-1 on genes that contain binding sites for only one class of factor, they do not account for the more complex pattern of regulation observed for genes containing composite response elements, as studied most extensively in the case of the proliferin gene. In this case, nuclear receptors and AP-1 protein can act either cooperatively or antagonistically, depending upon the identity and conformation of the nuclear receptor (i.e., glucocorticoid or mineralocorticocoid receptor) and AP-1 complex (Jun/Jun or Jun/Fos), bound to the composite element. It is possible that on such an element, some combinations of co-activators promote, while others inhibit, the recruitment of CBP. We and others have further found that the JAK/STAT pathway also requires CBP/p300 for function (Bhattacharya et al., 1996; Horvai et al., 1996).

The CBP/p300 Family as Functional Integrators of Diverse Signal Transduction Pathways

The demonstration that CBP is required for transcriptional activation by CREB, AP-1, STATs, and nuclear receptors, and that these factors compete with each other to interact with limiting amounts of CBP within the cell, suggests that CBP functions as a nuclear integrator of multiple signal transduction pathways. Because the antagonistic effects of nuclear receptors and AP-1 can be abolished by raising the levels of CBP, regulation of CBP expression is likely to be of critical importance in determining the transcriptional consequences of simultaneous

activation of multiple pathways. Indeed, evidence that CBP is physiologically maintained at a limiting concentration is strongly suggested by the observation that individuals heterozygous for a truncated CBP transcription unit exhibit severe developmental defects, the Rubinstein-Taybi syndrome (Petrij et al., 1995). In addition, one subtype of acute myeloid leukemia is caused by a chromosomal translocation event that results in an in-frame fusion of CBP with another protein, MOZ. The potential role of CBP in growth control is also reflected in its interaction with viral protein E1A and SV40 T antigen, which are known to have profound effects on regulation of cell cycle.

We have proposed that the CBP/co-activator complexes should be considered as "integrators", based on their role in determining the relative transcriptional responses of a specific target gene in the face of activation of multiple signaling pathways. Thus, while the cross-talk between signal transduction pathways could occur in the cytoplasm, at the level of kinase cascades, partitioning of key transcription regulators onto different target genes could provide a nuclear mechanism to integrate multiple signaling events at the transcriptional level.

So far, CBP has been demonstrated to be a required component in transcriptional activation events induced by multiple pathways, including cAMP, NGF, hormones, growth factors and cytokines. These factors all have diverse and even opposing effects on the regulation of proliferation and differentiation state of a variety of cell types. Thus it is conceivable that when cells receive multiple signals, CBP is a key element in integrating all the signals by determining which set of genes are to be activated, and the level of transcription. In this way, CBP plays a key in determining the proliferation or differentiation state of a cell by balancing the expression level of key genes. Thus, when monoclonal antibody against CBP/p300 was injected into fibroblasts, we found that growth was inhibited, and when injected into myoblasts, the differentiation process was stalled (Eckner et al., 1996). These data indicate that the integration function of CBP is critical in determining proliferation vs. the differentiated state of the cell.

The identification of CBP as an integral component of the activation complex for cAMP, growth factors, and nuclear receptors has led us to suggest and provide evidence for a molecular mechanism by which nuclear receptors, JAK/STAT factors, and other factors can ultimately inhibit a portion of AP-1 action, based on their requirement for the relatively low endogenous intracellular levels of the CBP/p300 family proteins by these classes of transcription factors. Alternatively, when sites for different families of transcription factors are present in one enhancer, CBP/p300 promotes synergy of these transcription units. In this model, CBP/p300 serves as the nuclear integrator of diverse signal transduction pathways, and becomes partitioned by alternative activation of specific transcription factors by ligand or by activation of distinct phosphorylation cascades. The relative abilities of co-activators to recruit limiting amounts of CBP into distinct integrator complexes could account for aspects of the multifactorial control of biological processes by multiple signal transduction pathways, including the regulatory actions of neurofibromin. Thus, the precise cohort of genes activated in a given cell would depend both on the relative activity of diverse signaling pathways, and the organization and relative affinity of the CBP/p300 complex on various DNA-bound factors that utilize CBP as a co-activator.

BODY

EXPERIMENTAL METHODS

Representational Difference Analysis

In order to initiate the search for relevant gene targets responsible for altered degrees of cellular proliferation resulting from alternative modulation of CBP accessibility by cellular transcriptional machinery due to altered intensity of the ras signaling pathway, as is suggested for mutations in NF1, or owing to reduced throughput by cAMP-induced CREB activity, as in the case of dominant-negative CREB, we have thoroughly examined several alternative strategies, including differential display and suppression subtractive PCR (Diatchenko et al., 1996), but we opted for a strategy that is both advantageous on a theoretical basis, and more effective, as supported by our preliminary data. This approach is based on adaptation of representational difference analysis (RDA) (Lisitsyn et al., 1993), a technique that we have previously modified and successfully utilized to obtain probes which permitted the positional cloning of the gene responsible for the Ames genetic dwarf mouse phenotype (Sornsen et al., 1996). In that case, we took advantage of restriction site polymorphisms between distantly related murine strains, and availability of pools of mice homozygous at only the "disease" locus.

The RDA technique is a PCR-based subtractive hybridization strategy in which DNA fragments (resulting from restriction enzyme digestion) present in one, ("tester") but not a second ("driver") DNA source are selectively and exponentially amplified. This procedure entails (1) the attachment of oligonucleotide linkers to the "tester" pool of fragments, and (2) the addition of an excess of the "driver" pool of fragments followed by (3) the denaturation, annealing, and PCR-mediated amplification of the resulting material, utilizing primers homologous to the linkers attached to the "tester" DNA fragments. The resulting amplified DNA products are referred to as an "amplicons". After the first round of amplification, linkers are removed from the "amplicon", new linkers are attached, and this material is then used as the "tester" pool in a second round of "driver"/"tester" annealing and amplification. Following three rounds of amplification, this technique yielded DNA fragments which provided the critical probes that permitted us to construct a contiguous series of overlapping BAC (bacterial artificial chromosome) and YAC (yeast artificial chromosome) clones spanning the Ames *df* mutation, ultimately allowing us to clone the *Prophet of Pit-1* gene which is responsible for the dwarfism associated with the Ames mouse (Sornsen et al., 1996).

We investigated the possibility of applying this procedure to identify critical targets of modified programmed patterns of gene expression due to altered CBP-mediated integration of intracellular signaling pathways. We realized that even by conservative estimates, RDA could theoretically be utilized in the identification of transcripts which exhibit 10-fold or greater differences in their concentration in two different sources. We have considered what levels of differential expression and abundance of a target gene one might expect to be able to identify with this technique, based on the estimate that 50% transcripts consist of ~15,000 rare mRNAs. If we assume that the expression of *bona fide* target gene may be reduced (or enhanced) only 10-fold in the absence of the POU domain factor, and furthermore, a particular target mRNA is ~10,000-fold less abundant than abundant mRNA species, we have calculated that it will still be possible to isolate RDA products corresponding to that target gene. If hybridization is performed using a driver/tester ration of 100:1, after two rounds of amplification, a rare target gene would become > 150-fold more abundant than even the most abundant gene. Therefore, many target genes can be isolated after two rounds of reannealing; however, three rounds of

reannealing would additionally enrich target genes another >100-fold, achieving an overall enrichment of > $5x10^5$-fold. Thus the RDA technique can be adapted to detect differential expression of even very rare transcripts. As described in the experimental results, the RDA technology as described above has been applied to cultures of Schwann cells and a variety of candidate genes have been described.

We have further refined the strategy to perform RDA with as little as 1 μg of total RNA, designing a "carrier", an RNA transcribed *in vitro* from a template that consists of a 528bp fragment of DNA from the Drosophila Antennapedia P1 promoter (Laughon et al., 1986) lacking sites for DpnII and NlaIII, and fused to an artificial polyA tract, so that while the polyA tract allows the carrier to be isolated as mRNA, the resulting cDNA cannot be cut with NlaIII or DpnII, avoiding linkering and amplification of the carrier during the RDA procedure. This refinement permits RDA to be successfully employed with tissues such as Schwann cells, which due to obvious constraints, are obtained in relatively small quantities from each individual, owing to the minute nature of the architecture of the peripheral sciatic nerve in mice. Following the third round of amplification (~ 10^5 selective enhancement) using the modified RDA technique, amplified cDNAs will be cloned and sequenced. Putative target genes will be characterized by comparing their sequences to databases, and by evaluating their expression level in wild type and mutant tissues using the "Snorthern" technique (Sornsen et al., 1996), and *in situ* hybridization. We developed the "Snorthern" procedure to provide amplified sets of fragments that assure almost complete coverage of cDNAs derived from restricted tissue sources (Sornsen et al., 1996). cDNAs that reflect specific induction (or repression) events will be subjected to further analysis, and full copy cDNAs for candidate transcripts will be isolated from a Schwann cell cDNA library. Currently, we are in the process of applying this modified RDA technique to an existing genetic paradigm, the Tst-1 (-/-) mouse, which is defective in the final stage of myelination (Bermingham et. al.), and is an ideal system for determining the applicability of this technique to the analysis of NF1 (-/-) and Ser133→A CREB Schwann cells relative to wild type cells.

Targeted Mutagenesis of the NF-1 Gene

The basic procedures for generating mutations in mice by homologous recombination are extensively utilized in our laboratory (Schoneman et al., 1995; Erkman et al., 1996; Bermingham et al., 1996; McEvilly et al., 1996). Briefly, the procedure consists of the following steps: 1) A targeting vector is built that is theoretically capable of undergoing homologous recombination with the gene to be disrupted. 2) Mutations are produced in embryonic stem (ES) cells in culture by homologous recombination of the target gene with the introduced DNA. 3) Mutant ES cells are introduced into host blastocysts that are in turn introduced into pseudopregnant mice and allowed to develop into chimeric mice. 4) The chimeric mice are bred to determine if the ES cells contribute to the germ line of the chimera. If so, mutations that they carry can be transmitted to future generations.

The symptoms of neurofibromatosis manifest themselves in humans who possess only one normal *NF1* allele, presumably due in part to secondary somatic mutations, yet mice heterozygous for either of the two currently available targeted *NF1* mutations (Branan et al., 1994; Jacks et al., 1994) fail to develop neurofibromatosis, which severely compromises their efficacy as an animal model of this disease. Homozygous mutant mice die in mid-gestation of cardiac defects, precluding their generation of neurofibromas *in vivo*. Whereas both currently available mouse *NF1* mutant alleles possess insertions of a neomycin resistance gene into exon 31 expressed in all cell types throughout development, we utilize the double-replacement strategy (reviewed in Ramírez-Solis and Bradley, 1994) to conditionally delete exon 31 in glial

cells around the time of birth using Cre recombinase under the control of a cell-specific promoter, resulting in a stop codon 9 amino acids downstream of the junction of exons 30 and 32, and the production of an NF1 protein truncated in the GAP homology domain, and thus incapable of interaction with the ras signaling pathway.

The double replacement strategy (Askew et al., 1993, Ramírez-Solis and Bradley, 1994), to be employed in the generation of both of the proposed *NF1* knockouts, involves an initial recombination event in cultured embryonic stem cells. Following this electroporation, ES cells are subjected to positive selection only, and the resultant ES cell colonies are then screened for homologous recombination events to isolate clonal cell lines which have inserted the positive and negative selectable marker genes. The resulting ES cell line "A" can be utilized to generate the Schwann cell specific conditional mutant "B" lacking exon 31, to be expressed only in Schwann cells. To generate the conditional mutation, the ES cell line "A" is transiently co-transfected with a plasmid that expresses a re-engineered Cre recombinase (kindly provided by J. Marth), designed to have an idealized Kozak sequence and a nuclear localization signal. ES cell lines isolated after this procedure that possess restriction fragment length polymorphisms characteristic of type II ("B") recombinants will be injected into blastocysts to generate mice that possess the type II mutation: the *NF1* exon 31 flanked by loxP sites.

Mice containing type "B" mutations are crossed to transgenic mice that express Cre recombinase in a tissue of interest. F1 offspring that are heterozygous for both the transgene and the type II *NF1* mutation will be backcrossed to mice that are homozygous for the type II deletion. 25% of the progeny from this backcross will be homozygous for the type II mutation, and will express the P_0-Cre transgene. In these mice, Cre recombinase will generate a type I mutation, selectively deleting exon 31 in Schwann cells. There, Cre recombinase excises sequences between the two remaining loxP sites, inactivating the gene only in that tissue.

We plan to express Cre recombinase in Schwann cells of transgenic mice by utilizing mice which express this transgene under the control of the myelin P_0 protein. 1.1kb of P_0 regulatory sequences have been used successfully to target expression of transgenes to the mouse Schwann cells (Messing et al., 1992), and transgenic mice that express Cre recombinase under control of the P_0 promoter are currently available to us.

Dominant-Negative CREB Transgene

The experiment involves expression of the Ser133→A dominant-negative CREB in peripheral myelinating glia using the P_0 promoter (Messing et al., 1992). 1.1kb P_0 promoter, fused to globin exon 1-2 (noncoding exons)/intron region, to provide a 5' splice site, is used to drive the Ser133→A CREB transcript using hGH 3' untranslated/poly A signal as we have successfully employed in previous transgenic cDNA experiments (Rhodes et al., 1993, Treier et al., 1998). The transgene released as a Not-1 fragment is purified and microinjected into the (C57BL/6J x DBA/2J) fertilized eggs (Rhodes et al., 1993; DiMattia et al., 1996). Eggs surviving microinjection are transferred to (C57BL/6J x DBA/2J) F1 pseudopregnant female recipients. Transgenic mice will be identified by PCR and Southern blot analysis, and at least three pedigrees will be established.

Microinjection of Cultured Schwann Cells

While these experiments are designed to ultimately provide a platform for the evaluation of putative target identified from Schwann cell populations by RDA, our initial goals are directed towards the establishment of conditions suitable for the growth, maintenance, and utilization of these Schwann cell populations in the microinjection assay. Schwann cell cultures

will be prepared essentially as described previously (Kioussi et al., 1994). Briefly, the sciatic nerve from 14.5 d.p.c., 1, and 5 day old mice are dissected, and incubated in 400µl enzyme solution containing collagenase (0.3%) for 1 hour in L-15 medium + 10% fetal calf serum (FCS) at 37°C. After incubation, the sciatic nerves are rinsed 3-4 times in L-15/FCS, resuspended in DMEM/HAMS F 12 containing trace nutrient and hormonal supplements and 10% fetal calf serum (Kioussi et al., 1994), counted and plated at a density of 3000 cells per coverslip (1.6 cm in 24 well tissue culture dishes (Nunc) in a 30µl drop on poly-L-lysine/laminin-coated acid washed glass coverslips. Alternatively, for longer term cultures, the cells are plated onto plastic coverslips (Sarstedt) which have been coated previously with uv-/ammonium hydroxide-crosslinked rat tail collagen. After 1.5 h, cultures are covered with 1 ml of medium. For long term dissociated Schwann cell cultures, 24 h after plating the cells are treated with 10^{-5} M cytosine arabinoside for 24 h to kill dividing cells and the cells are grown in serum free medium. When indicated, forskolin (2 µM), a reversible activator of adenyl cyclase, or 8-Br cAMP (2 x 10^{-3}M), or specific growth factors will be added to cultured cells for 48 hours in order to induce proliferation. The proliferation state of cultured glial cells can be monitored with BrdU staining, and the expression of p21 gene with immunostaining. Normally, when glial cells are treated with cAMP, BrdU will be incorporated and p21 expression will be extinguished.

Prior to microinjection, the cells are rendered quiescent by incubation in serum-free medium for 24-36 hours. Plasmids or antibodies are injected into the nuclei of cells at a final concentration of 100 µg/ml. Either preimmune rabbit IgG or specific affinity-purified IgG antibody are co-injected to later allow the unambiguous identification of the injected cells. Microinjections are carried out using an Eppendorf semiautomated microinjection system mounted on an inverted Zeiss microscope. Approximately one hour after injection, the cells are stimulated with the appropriate agents. After overnight expression, the cells are fixed with 3.7% formaldehyde and then stained to detect injected IgG and where appropriate, β-galactosidase expression (Kamei et al., 1996). The latter are detected by incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Injected cells will be identified by staining with tetramethylrhodamine-conjugated donkey anti-rabbit IgG. Cells will be viewed and analyzed on a Zeiss Axiophot microscope. Photomicrographs will be taken with the same equipment using Kodak Ektachrome 400 color film.

RESULTS

Representational Difference Analysis

As a "proof of principle", we have elected to apply representational difference analysis (RDA) to isolate cDNAs that are differentially expressed in two distinct model systems. The first of these utilizes cultured Schwann cells and has the advantage of supplying relatively large amounts of material for this type of analysis, and permitted us to test the system without the limitations imposed by limiting amounts of starting material. Having developed the methodology successfully in this system, as described below, we are currently involved in the adaptation of this technique to the second model system, which exploits the comparison of the Tst-1 null mutation, in which the Schwann cells have arrested at the stage of the initial wrap of myelination, failing to progress to the ensheathment stage, with the Schwann cells from wild-type mice. We reasoned that the successful implementation of RDA in this next step will demonstrate that the otherwise limiting amounts of material available from sciatic nerves will be

sufficient for the application of the RDA technology to the comparisons of tissues from otherwise isogenic mouse strains. Preliminary indications from our experimental analyses suggest that this application of the RDA technique will equal or exceed or expectations.

In the initial model system which we have thus far completed our analyses, Schwann cells were grown in the presence or absence of forskolin, an agent that raises intracellular levels of cAMP and mimics many aspects of terminal differentiation in these cells [224]. Total RNA was isolated from cultured cells or from tissues using Trizol reagent (Gibco), and using the assumption that 1 μg of total RNA contains 20 ng of poly A+ mRNA, carrier mRNA was added to bring the total amount of mRNA to 200 ng. The mRNA was isolated from the total RNA samples using Oligotex beads (Qiagen), and the cDNAs were synthesized using the Superscript cDNA synthesis kit (Gibco). Both cDNA samples were digested separately with DpnII and NlaIII. Linkers were ligated onto the ends, and fragments that are 40- 1000 base pairs in length and contain linkers at each end were amplified by PCR to generate approximately 20 μg of cDNA, after which the linkers were removed. New linkers were added to 500 ng of each amplified cDNA sample to generate the tester samples, the remainder constituting the drivers. 5 μg of driver was mixed with 50 ng of tester derived from the other sample, denatured and allowed to reanneal to a Cot of 300. Typically, genomic DNA re-associates at Cot 100-1000; but because cDNA is less complex, we expected the majority of cDNA fragments would reanneal under these conditions. After two rounds of denaturation and reannealing, distinct bands were visible. Thus far we have isolated and examined 48 clones that corresponded to 45 potential target genes. Of these, 26 differentially hybridized to amplified cDNA derived from forskolin treated or untreated Schwann cell RNA, indicating that they may indeed be directly or indirectly activated or repressed by increased levels of intracellular cyclic AMP. Among these genes were CD9, a protein known to affect Schwann cell morphology [215,216], and two homologues of latent TGF-β binding protein that modulates the response to TGF-β. Because TGF-β inhibits myelination in vitro [217] these results suggest that the rise in intracellular cAMP at the onset of Schwann cell differentiation serves to inhibit TGF-β signaling, thereby permitting myelination to proceed. A detailed accounting of target genes evaluated in this procedure is presented in Table I.

Construction of Targeted Recombination Vector

We have devised a complete strategy in progress to generate targeted recombination vectors for disruption of the NF1 gene locus in the mouse as follows: The isolation of overlapping genomic fragments from genomic DNA isolated from a mouse lambda FixII genomic library constructed from the 129/sv mouse strain (Stratagene) extending from exon 29 to exon 33, encompassing 14 kb of the mouse NF1 locus (Fig. 1). The backbone vector for the initial construction of the conditional recombinant vector is pFlox (provided by J. Marth) (Fig. 2). The strategy for the assembly of this vector currently involves the insertion of a Hind III-Eco RI 7.7 kb fragment inserted into the unique Hind III site, an Eco RI- Pst I 1 kb fragment inserted into the unique Bam HI site, and a 1.3 kb Pst I-Pst I fragment inserted into the unique Sal I site of the pFlox vector (Fig. 3). The construct will be linearized at the unique Not I site prior to electroporation.

Strategies for the Identification of Homologous Recombinant ES Cell Lines

We have applied our analysis of the *NF1* locus to design strategies and create probes for the identification of ES cell line clones which have successfully undergone targeted homologous recombination at the *NF1* locus. Two probes corresponding to portions of the *NF1* locus which are not part of the targeted recombination vector are utilized for the screening of

embryonic stem cell lines in southern blot hybridization analysis. The upstream probe is derived from PCR amplification of exon 29, and is approximately 300 bases in length. Following Pst I digestion of genomic DNA derived from ES cell line clones obtained following the initial electroporation with the conditional recombinant vector, the radiolabelled exon 29 fragment hybridizes to a band of 12 kilobases corresponding to the wild-type *NF1* allele, and in the event of targeted homologous recombination, the exon 29 fragment hybridizes to a 12.5 kilobase fragment corresponding to the mutant allele. To confirm recombination on the downstream side of the *NF1* gene, a probe derived from PCR amplification of exon 33, approximately 200 bases in length, is utilized in conjunction with Eco RI digested genomic DNA from ES cell clones. The band corresponding to the wild-type allele will be 3.5 kilobases and the band corresponding to a homologous recombination event yields a 2.5 kilobase fragment. To confirm the absence of additional insertions of the conditional homologous recombinant vector, a Pst I fragment derived from the neomycin gene of the targeting vector is hybridized to Eco RI digested genomic DNA for positive clones and the expected band of 2.5 kilobases is the only band observed for these clones. A secondary examination is also possible, utilizing a Hind III digest of genomic DNA and hybridization with the neomycin Pst I fragment. In this case the only observed band is 12 kilobases.

Following the identification of ES cell lines harboring the desired homologous recombinbant (Fig. 4), they are subsequently separately subjected to transient co-transfection with a plasmid encoding Cre recombinase in combination with negative selection against the HSV thymidine kinase gene present in the mutated *NF1* allele. From each of these original cell lines, clonal cell lines are selected which have survived the negative selection and genomic DNA prepared from them for analysis. At this stage it is necessary to screen these cell lines for Type II recombinants, which have deleted the drug resistant cassette, but have left in place Cre loxP sites flanking exon 31 (Fig. 5). To identify these clones, they are screened with three separate probes in subsequent southern blot hybridizations with appropriately digested genomic DNAs. The first of these consists of a Pst I digest combined with hybridization with the probe derived from exon 29. The successful recombinant will have a mutant band of 13.3 kilobases, as well as a wild-type band of 12 kilobases. The second analysis utilizes a probe derived from exon 33 and an Eco RI digest. A correct Type II recombinant will demonstrate an 11.2 kilobase mutant band as well as a 3.5 kilobase wild type band. Hybridization of either Pst I or Eco RI digested genomic DNA should not yield any band, as these sequences should have been excised from the locus.

Because Type I recombinants, in which the drug resistant markers and exon 31 are deleted, are also likely to be produced during the transient co-transfection described above, we have also designed a strategy to identify these types of clones. In these cases, hybridization with the exon 29 probe described previously would generate a mutant 12.3 kilobase band, rather than the band of 13.3 kilobases, as well as the wild-type band of 12 kilobases. For the exon 33 probe, a mutant 10.2 kilobase band would be generated, instead of the mutant 11.2 kilobase band described above for the Type 2 mutant, as well as a wild-type band of 3.5 kilobases corresponding to the unaffected allele.

Construction of Transgenic Dominant Negative CREB Construct

Primers corresponding to first and last eight amino acids of rat CREB cDNA flanked with Hind III and Eco RI restriction sites at the 5' and 3' ends, respectively, were utilized to PCR amplify an insert containing the entire rat CREB cDNA sequence which was subcloned into pBKS following restriction digest of the insert. The integrity of the sequence was confirmed by standard DNA sequencing methodologies. The sequences surrounding serine 133 were utilized to introduce an alanine at this position with a T to G substitution in the first

position of codon 133 utilizing site directed mutagenesis (Stratagene). The fragment is being excised with Eco RI and Hind III, blunt ended, and inserted at the Sma I site in the TR3 transgeneic vector (Rhodes et al., 1993; DiMattia et al., 1996; Treier et. al., 1998) between the 5' betaglobin intron and the 3' poly adenylation cassette. This cassette will then be removed by Sal I and Cla I restriction digestion, blunt ended, and inserted into a blunt ended Asp 718 site in pBKS. Finally, the 1.1 kb rat P0 promoter will be removed by Hind III digestion from pPG6 (gift of D. Weinstein), blunt ended, and inserted into pBKS/TR3/CREB which has been digested and blunt ended at the Cla I site in the polylinker upstream of the 5' betaglobin intron. Orientation following each of these steps is accomplished by DNA sequencing. Prior to microinjection P0 promoter-CREB insert is removed from the pBKS vector by Not I digestion and purified for injection as described (Rhodes et al., 1993; DiMattia et al., 1996; Treier et. al. 1998).

Screens for Interaction partners of CBP

Utilizing a variety of techniques, we have initiated screens for combinatorial complexes which interact with CBP under a variety of cellular conditions in response to diverse extra- and intracellular stimuli. These studies include specific mutagenesis of interaction domains including the LXXLL motif initially observed in CBP and associated factors, and will serve to discriminate between the various domains of CBP which interact simultaneously with coregulators and those which act through mutual exclusivity. These studies are ongoing, utilizing the microinjection technology as described in the methods section and in the main body of the Grant, and conclusions pertaining to the exact representational natures of the interaction domains will be forthcoming in the next year.

Microinjection of Cultured Schwann Cells

We have established culture conditions, described in the experimental methods section, for the establishment of Schwann cell cultures which can be adapted to a microinjection strategy. To demonstrate the efficacy of this technique we have microinjected rabbit IgG together with a betagalactosidase expression vector under the control of the position independent CMV promoter, and prepared the resulting samples for photography as described in the experimental methods section. The data as shown in figure 6, document our ability and proficiency in the adaptation of a microinjection strategy to the analysis of primary cultures of Schwann cells. The cells were fixed for analysis one day after microinjection to allow for expression from the reporter plasmid. Panel A demonstrates representative staining of an injected Schwann cell with tetramethylrhodamine-conjugated donkey anti-rabbit IgG utilizing fluorescent microscopy. Panel B demonstrates expression of the betagalctosidase reporter plasmid microinjected into in this Schwann cell as assayed by incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal).

DISCUSSION

As outlined under Experimental Methods and Results, we have made significant progress in our goals as described in the specific aims of this Grant. We have implemented a strategy to establish animal models of NF1 through the construction of recombinant vectors designed to replace the NF 1 gene with a nonfunctional version of this gene and have established a means by which ES cell lines harboring the correct mutations can be identified. In further support of this objective, we have made a significant technological advance in our

capacity to functionally evaluate the phenotype of Schwann cells through our successful implementation of the representational difference analysis (RDA) technique in cultures of Schwann cells, and anticipate that in the near future we will complete the extension of this technology to the analysis of Schwann cells derived from sciatic nerve, which will enable us to characterize the consequences of misregulated programmed patterns of gene expression in *NF1* mutant mice. Our analysis of the misregulation of neurofibromin-regulated signal transduction at the nuclear level through altered availabilities of the transcriptional integrator CBP have progressed with the near completion of the construction of a dominant negative CREB transgene, with ongoing analysis of the means by which distinct regulatory motifs present in CBP and in competing cofactors associated with CBP determine the relative accessibility of CBP for the interaction with core transcriptional machinery, and with the establishment of culture conditions for Schwann cells which enable microinjection technology to be utilized in the experimental manipulation of these cells.

CONCLUSIONS

Our efforts are focused towards an understanding of the molecular mechanisms underlying the uncontrolled Schwann cell proliferation that characterizes the etilology of the human disease neurofibromatosis type I. A critical aspect of our understanding of this disease requires the ability to evaluate the alterations in gene expression following the inactivation of crucial regulators of signal transduction such as the product of the *NF1* gene, neurofibromin. Although Neurofibromatosis is inherited as an autosomal dominant trait, the preponderance of data in the scientific literature suggest that the mechanism is itself recessive in nature, acting, as so have been many tumor suppressors been shown to do, when, in individual cells, the second, normal, allele undergoes somatic mutation. We have hypothesized that the subsequent abnormal proliferation of Schwann cells is due to improper integration of intracellular signal transduction pathways including cAMP- and ras-related signaling at the nuclear level, with CREB and AP-1, respectively, by competition by these and other factors for limiting amounts of the nuclear integrator CBP, which in turn regulates transcription of programmed patterns of gene expression through contact with the core transcriptional machinery. The ability to evaluate differences in these programmed patterns of gene expression is a crucial component of any effort to identify the factors responsible for directing uncontrolled proliferation of Schwann cells. We have successful developed and implemented a representational difference analysis (RDA) technique and utilized this approach to identify target genes in Schwann cells which are either upregulated or downregulated in response to various perturbations of the intracellular signaling pathways under investigation in this project. This technical advance is currently being applied to isolated Schwann cells from wild type and Tst-1 (-/-) mice, the later of which display a defect in myelination, in order to demonstrate the applicability of this technique to small quantities of tissues such as sciatic nerve, a highly enriched source of Schwann cells. Our successful implementation of the RDA technique now enables us to identify candidate target molecules which are misregulated in the varieties of Schwann cells we are in the process of creating, and thereby will suggest candidate molecules responsible for the abnormal growth patterns of Schwann cells. The combination of this process, which will suggest candidate gene products to be evaluated in Schwann cell proliferation assays, together with our continuing approach to the elucidation of CBP function by the analysis of CBP interaction partners, will serve to advance our knowledge of the mechanisms by which CBP integrates intracellular signaling as well as our understanding of the nature of the different classes of target genes these signaling pathways regulate, and ultimately suggest factors to which pharmacological approaches of treatment may ultimately be devised.

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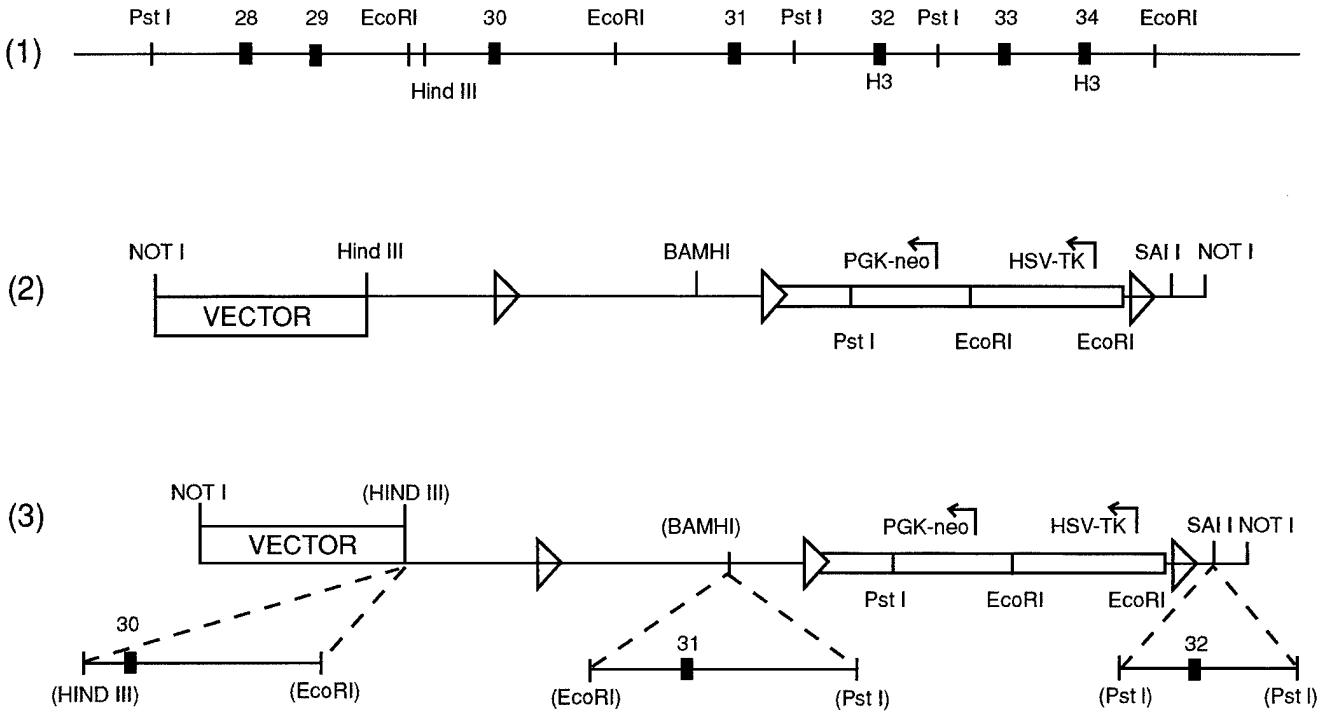
APPENDICES

Figures 1-5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(BAMHI)
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EcoRI
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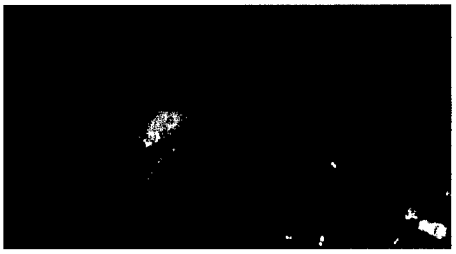
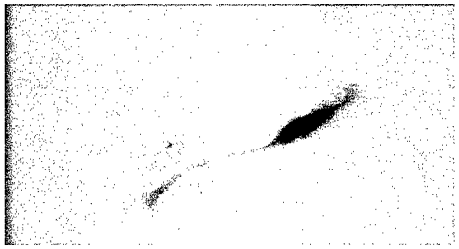
H3
H3
(5)
Pst I
28
29
EcoRI
30
(EcoRI)
(Pst I)
32
Pst I
33
34
EcoRI
Hind III
H3
H3

Figure 6

(A)



(B)



Clones from RDA using RNA from Schwann cells cultured with or without forskolin

clone	seq	Size	Forskolin regulation	homology	P(N)
1 (1-1)	T7T3	164bp	**up**	latent TGFβ binding protein-like; without intron	5.7e-32
2 (1-2)	T7	325bp	**up**	EST H34321 similar to cerebellin-like glycoprotein	8.9e-37
3 (1-3)	T7	325bp	up	EST H34321 same as 2	8.9e-37
4 (1-4)	T7T3	115bp	**up?**	unknown sequence	
		44bp	**up?**	unknown sequence; same as 22	
		167bp	**up?**	EST AA648722	1.1e-48
5 (1-5)	T3	189bp	up	EST AA207500; same as 66	1.6e-52
6 (1-6)	T7	262bp	up	EST W51468	1.6e-83
7 (1-7)	T7T3	232bp	**up?**	unknown sequence; same as 51	
		336bp	**up?**	latent TGFβ binding protein-like; with intron; same as 65	2.1e-77
8 (1-8)	T7T3	253bp	**up**	Myelin and Lymphocyte Protein (MAL); X82557: 1535-1787	8.8e-97
9 (1-9)	T7T3	166bp	**up**	Myelin and Lymphocyte Protein (MAL); X82557: 1373-1538	2.8e-61
10 (1-10)	T7T3	171bp	**up?**	CART1 (RING finger protein)	9.0e-45
		289bp	**up?**	mcd39L1 (ecto ATPase) U91511: 1408-1476; 1408-1641; 1658-1693	3.0e-70
11 (1-11)	T7T3	254bp	**up?**	Myelin and Lymphocyte Protein (MAL); X82557: 1545-1787	1.3e-92
		336bp	**up?**	Myelin and Lymphocyte Protein (MAL); X82557: 466-596; 650-801	1.0e-104
		223bp	**up?**	EST AA346230	1.4e-35
12 (1-12)	T7	253bp	up	Myelin and Lymphocyte Protein (MAL); same as 8	8.7e-97
13 (2-1)	T7	280bp	**down?**	unknown sequence	
		295bp	**down?**	Annexin VI (Ca^{2+}, phosphlipid binding)	1.2e-102
14 (2-2)	T7	196bp	down	18s rRNA?? M29839: 393-417; 467-540	1.3e-08
15 (2-3)	T7	281bp	**down**	18s rRNA M29839: 324-604	2.6e-110
16 (2-4)	T7	346bp	**down?**	K+ channel M81784: 816-1161	1.7e-135
		272bp	**down?**	rRNA	5.7e-101
17 (2-5)	T7	<367bp	down	collagen XIV	9.2e-17
18 (2-6)	T7	301bp	down	EST W53158	1.7e-87
19 (2-7)	T7	235bp	down	transferrin D38380: 344-567	6.2e-84
		246bp	down	EST AA420321	4.5e-75
20 (2-8)	T7T3	455bp	**down?**	RRP22 (ras superfamily member)	9.2e-125
		303bp	**down?**	KIAA0013 D87717: 2249-2369	1.5e-22
21 (2-9)	T7	368bp	down	K+ channel M81784: 458-694	3.7e-143
22 (2-10)	T7	44bp	down	unknown sequence; same as 4	
23 (2-11)	T7	346bp	**down**	18s, 5.8s rRNAs V01270: 5317-5531	1.6e-128
24 (2-12)	T7T3	243bp	**down**	G protein β2 subunit	1.2e-93

clone	seq	Size	Forskolin regulation	homology	P(N)
25 (3-1)	T7	291bp	up	EDG-3 (G protein coupled receptor)	2.8e-86
26 (3-2)	T7	289bp	up	extracellular SOD X68041: 360-648	8.8e-113
27 (3-3)	T7	322bp	up	semaphorin	2.6e-98
		158bp	up	EST R62197	1.3e-20
28 (3-4)	T7	185bp	up	vimentin M24849: 522-551; 568-622; 651-700	2.2e-35
		53bp	up	vimentin M24849: 713-764	2.6e-12
29 (3-5)	T7	243bp	up	EST AA068111	5.0e-90
30 (3-6)	T7	316bp	**up**	CD9 (tetraspan protein)	1.1e-125
31 (3-7)	T3	247bp	up	collagen I	3.0e-71
32 (3-8)	T3	261bp	up	68kd neurofilament protein	5.0e-91
		<602bp	up	vimentin X62952 (M24849: 897-1145)	2.6e-106
33 (3-9)	T7	281bp	up	18s rRNA M29839: 324-604	1.5e-109
34 (3-10)	T7	198bp	up	HSP90 heat shock protein	2.5e-69
		345bp	up	18s, 5s rRNAs V01270: 5187-5435; 5415-5531	7.4e-123
35 (3-11)	T7	281bp	up	18s rRNA M29839: 324-604	1.5e-109
36 (3-12)	T7	247bp	up	EST T07169 same as 86	7.3e-43
37 (4-1)	T7	456bp	**down?**	brain hexokinase	4.7e-172
		149bp	**down?**	agrin precursor	1.7e-42
38 (4-2)	T7	210bp	down	unknown sequence	
39 (4-3)	T7	264bp	down	X. laevis LDL receptor	2.5e-05
40 (4-4)	T7	269bp	down	EST W10180	3.3e-32
		220bp	down	unknown sequence; same as 70, 71	
41 (4-5)	T7T3	153bp	**down?**	insulin-1 (TCR-β complex?)	4.9e-51
		244bp	**down?**	EST AA241341	1.5e-69
		246bp	**down?**	H36-α7 integrin	1.3e-77
42 (4-6)	T7	281bp	**down**	18s rRNA M29839: 324-604	2.7e-110
43 (4-7)	T7	219bp	down	Multidrug resistance associated protein (plasma membrane efflux pump)	2.5e-59
44 (4-8)	T7	228bp	**down**	actin binding protein X53416	6.3e-37
45 (4-9)	T7T3	222bp	down	OR-1 orphan nuclear receptor	1.2e-84
46 (4-10)	T7	366bp	down	unknown sequence	
47 (4-11)	T7T3	154bp	down	βig-h3 (TGF-β inducible ECM protein)	4.9e-51
48 (4-12)	T7	223bp	**down**	PP2A protein phosphatase (65kDa)	4.8e-70

clone	seq	Size	Forskolin regulation	homology	P(N)
49	T7T3	163bp	up	latent TGFβ binding protein-like; without intron	2.4e-41
		256bp	up	EST W84171	1.4e-07
50	T7	303bp	up	mcd39L1 ecto ATPase U91511: 1108-1384; 1386-1411	9.5e-91
51	T7	232bp	up	unknown sequence	
52					
53	T7	291bp	up	EST W10995	2.6e-19
54	T7T3	253bp	up	Myelin and Lymphocyte Protein (MAL) same as 8	1.6e-97
55	T3	213bp	up	KIAA0005	6.2e-41
56	T7	91bp	up	mcd39L1 U91511: 1043-1111	2.2e-10
		54bp	up	mcd39L1 U91511 889-932	5.7e-06
57	T7T3	253bp	up	Myelin and Lymphocyte Protein (MAL) same as 8	1.5e-97
58	T7	762bp	up	furin (propeptide processing protease)	4.3e-204
59	T7T3	234bp	up	N10 (NGF-induced glucocorticoid receptor-like gene)	2.4e-87
		198bp	up	unknown sequence	
		290bp	up	mcd39L1 U91511: 1408-1502; 1640-1693	1.4e-77
60	T7	163bp	up	latent TGFβ binding protein-like, without intron	2.7e-22
61	T7	253bp	up	MAL (same as 8)	1.5e-97
62	T7T3	253bp	up	Myelin and Lymphocyte Protein (MAL)	1.7e-95
		230bp	up	tyrosine phosphatase	3.4e-87
63	T7	261bp	up	68kd neurofilament protein gene	4.9e-91
64	T7	315bp	up	neurotrypsin	1.4e-104
		592bp	up	mcd39L1 (ecto ATPase) U91511: 1432-1550; 1647-1693	1.4e-34
65	T7T3	336bp	up	latent TGFβ binding protein-like; with intron	9.5e-21
66	T7	188bp	up	EST AA207500; same as 2	6.3e-49
67	T7	372bp	down	prosaposin S81373: 568-855	4.9e-130
68	T7	302bp	down	EST W77252	1.9e-27
		253bp	down	dystroglycan U43512: 463-504; 508-576; 536-710	6.5e-60
69	T7	232bp	down	transferrin D38380: 404-517; 512-577	2.5e-80
70	T7	220bp	down	unknown sequence; same as 40, 71	
		361bp	down	KIAA0013 D87717: 3146-3477	4.1e-80
71	T7	466bp	down	leucocyte adhesion glycoprotein M81695: 1064-1327	4.7e-72
			down	unknown sequence; same as 40, 70	
72	T7	323bp	down	dystroglycan U48854: 190-220; 220-464; 448-489; 486-511	1.7e-108

clone	seq	Size	Forskolin regulation	homology	P(N)
73	T7	281bp	down	18s rRNA M29839: 324-604	4.7e-111
74	T7	343bp	down	28s rRNA	6.5e-115
75	T7	241bp	down	leucocyte adhesion glycoprotein M81695: 821-1000; 1005-1059	1.5e-48
76	T7	268bp	down	Ulip (Unc-33-like phosphoprotein; axonal guidance)	8.5e-95
77	T7	269bp	down	leucocyte adhesion glycoprotein M81695: 822-843; 864-940; 987-1059	7.4e-25
78	T7	378bp	down	EST AA637675 (also 18s,5s rRNAs V01270: 5453-5378; etc.)	1.0e-47
		<403bp	down	unknown sequence	
79	T7	267bp	down	leukocyte adhesion glycoprotein M81695: 1060-1257; 1260-1327	1.5e-69
		370bp	down	orphan G protein coupled receptor	2.1e-144
80	T7	374bp	down	Gallus gallus p52 protein (SH3-domain protein)	1.9e-41
		224bp	down	Gallus domesiticus filamin	9.7e-08
81	T7	207bp	down	prosaposin S81373: 568-774; alternative splice?	6.5e-77
82	T7	412bp	down	EST AA349394	2.8e-26
		150bp	down	unknown sequence	
		231bp	down	unknown sequence	
83	T7T3	223bp	down	EST AA139804	7.6e-82
		286bp	down	EST AA153559	2.5e-49
		304bp	down	KIAA0013 D87717: 2249-2369	1.5e-22
84	T7	300bp	down	APB-280 (filamin)	2.6e-35
85	T7	106bp	up	mitochondrial adenine nucleotide translocator	2.3e-36
86	T7	247bp	up	EST T07169	8.1e-43
		281bp	up	18s rRNA M29839: 324-604	8.5e-109
87	T7	121bp	up	EST AA306344: 250-364	7.7e-37
		62bp	up	EST AA306344: 194-245	2.0e-08
88	T7	284bp	up	extracellular SOD X68041: 360-529	2.8e-90
89	T3	258bp	up	P0	5.8e-37
		281bp	up	18s rRNA M29839: 324-604	4.8e-108
90	T7	350bp	up	EST AA240567	1.2e-111
		256bp	up	SR13/PMP-22	3.5e-79
91	T7	247bp	down	EST AA272137	2.6e-82
		214bp	down	β-actin	3.3e-81
92	T7	289bp	down	EST W66776	1.5e-106
93	T7	194bp	down	MAP1B microtubule-associated protein	4.4e-42
		231bp	down	ezrin	1.7e-66
		211bp	down	EST T39504	1.1e-63
94	T7	270bp	down	SCA7 (spinocerebellar ataxia 7)	1.3e-16
		282bp	down	18s rRNA M29839: 325-604	7.1e-110
95	T7	257bp	down	EST W85651	4.7e-18
96	T7	327bp	down	EST AA396161	3.4e-107

underlined numbers: Qiagen preps made; **bold up/down**: "confirmed" on dot blot; P(N): probability that sequence match is random